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(54) Title: HEPATOCYTE TEST KIT

(57) Abstract

An in vitro test kit is provided that comprises primary hepatocytes plated in crosslinked collagen-coated housings and cultivated in media comprising Chee's Essential Media supplemented with glutamine, arginine, thymidine, sodium bicarbonate, gentamicin, insulin, transferrin, selenium acid, dexamethazone and DMSO; a method for using the test kit for assessing liver morphology, physiology, and pathophysiology is also provided.

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HEPATOCYTE TEST KIT

BACKGROUND OF THE INVENTION:

This invention relates in general to mammalian cell cultures and more specifically to a hepatocyte test kit for morphological, physiological and pathophysiological testing.

The primary goal of most in vitro systems is to obtain results that duplicate the results that one would achieve in vivo. Much of the current biological research is, therefore, concerned with duplicating, or closely replicating, in vivo processes in vitro.

At the present time, most studies directed to liver function, including hepatotoxicity, are conducted on whole animals. However, alternatives are being sought for humane and financial reasons. A primary alternative of particular value would be a hepatocyte in vitro test kit which mimics in vivo conditions, wherein the hepatocytes are well characterized, healthy, highly differentiated, and have morphology and function of hepatocytes in vivo.

The liver serves many functions, among them macromolecular synthesis, energy generation and storage, catabolism, and biotransformation of toxic substances and waste products of intermediary metabolism. The hepatic parenchyma is comprised of three major cell types: hepatocytes, biliary epithelial cells, and Kupffer cells. Hepatocytes comprise about 80 percent of total liver mass. The biochemical

function of the liver includes the metabolism of amino acids, ammonia, proteins, carbohydrates, and lipids, biochemical oxidation, and the metabolism and detoxification of drugs, vitamins, and hormones.

Current liver testing has led to the following generalities. Since the liver plays a major role in generalized metabolic events, acute or chronic liver dysfunction in many instances leads to biochemical abnormalities. It has also been found that the liver has a large reserve capacity. Therefore, minor or moderate dysfunction often goes undetected with certain metabolic tests. Specifically, liver function tests based on biosynthesis or metabolic activity are of limited value. Lastly, some functions of the liver are more sensitive to injury than others, making detection and thus prevention difficult if not impossible.

The liver performs many varied functions, and no one test measures total liver function. Therefore, an understanding of the many known biochemical and metabolic functions of this organ is a prerequisite to identifying, analyzing and hopefully preventing or treating chronic or acute liver failure. Thus, it is a general objective of the present invention to gain insight into the biochemical and metabolic functions of the liver and effect of drugs on chemicals thereon.

In addition, it is a primary objective of the present invention to produce an in vitro test kit which reduces animal testing and is of predictive value to humans.

A further objective of the present invention is to establish a hepatocyte test kit which assesses liver physiology.

A still further objective is to provide a test kit which assesses liver pathophysiology. According to the Federal Register, more than 70,000 chemical compounds were commercially introduced in 1978, and between 700 and 3,000 new chemicals are introduced each year. From these only 6,000 were subjected to toxicity studies and then only to animals. Moreover, over 60,000 chemicals remained untested in animals or humans. This statistic highlights the necessity for a kit which ascertains toxins and/or toxic values in the liver and yields results which are applicable to humans.

A still further objective is to provide for a liver test kit which detects slight pathophysiological changes, wherein the hepatocytes are highly differentiated in morphology and function so that slight morphological and/or physiological changes are detectable.

A still further objective is to provide for a test kit which comprises long-term surviving hepatocytes.

The currently available hepatocyte culture media comprise synthetic formulations including glucose, salts, amino acids, vitamins, growth factors, etc. The major drawback of hepatocytes culture systems based on these simple media, is that the hepatocytes do not survive more than two days in vitro. By survival, we mean maintenance of in vivo morphological expression

and/or differentiated function. Without in vivo morphology and function, results are less significant.

A still further objective is to provide for a hepatocyte test kit capable of testing a wide variety of compounds.

A still further objective is to provide for a hepatocyte test kit which is sensitive to direct and indirect hepatotoxins.

Prior art attempts to increase the survival of cultivated hepatocytes include improving the culture medium or the substratum of the culture dishes, as described in U.S. Patent 4,914,032 and 5,030,105, both issued to Kuri-Harcuch, et al. These patents disclose hepatocyte cells at a density from about 180 to about 65,000 cells/cm^2 in a culture medium containing from about 1 to about 100 ug/ml of hydrocortisone, in the presence of either fibroblast cells treated to prevent their multiplication and to maintain normal morphology and expression of hepatocyte function for long periods, or fibroblast cell products on a modified substrate, at controlled densities from about 30,000 to about 65,000 cells/cm^2. The drawback of such a system is that more than one cell type is present and the test of experimental results are therefore more difficult to interpret and less consistent.

Another approach comprises the use of Matrigel, a basement membrane-like mixture, in the medium. Unfortunately, cells are difficult to harvest from the gel and Matrigel is very expensive or laborious to

obtain. Moreover, Matrigel may differ from lot to lot and as such is difficult to consistently reproduce.

A cell isolation technique comprising the use of an enzyme, collagenase, crude or purified, which produced a suspension of cells which were 95% intact, was disclosed in Seglen, P.O. Experimental Cell Research 82:391-398, 1973.

Basal culture conditions and more specifically the induction of cytochrome P-450 were disclosed in Waxman, D.J., Morrissey, J.J., Naik, S. and Jauregui, H.O. Biochemical Journal 271:113-119, 1990, and in Jauregui, H.O., NG, S.-F., Gann, K.L. and Waxman, D.J., Xenobiotica 21(9):1091-1106, 1991.

Dimethylsulfoxide (DMSO) as a culture additive was disclosed in Mukkassay-Kelly, S.F., Bieri, F., Waechter, F., Bentley, P. and Staubli, W., Experimental Cell Research 171:37-51, 1987. Isom, H.C., Secott, T., Georgoff, I.k Woodworth, C. and Mummaw, J., National Academy of Sciences USA 82:13252-3256, 1985, and Villa, P. Arioli, P. and Guitani, A., Experimental Cell Research 194:157-160.

In vitro liver test systems have been described by Guillouzo, A. Hepatotoxicity, In Vitro Toxicity Testing: Applications to Safety Evaluation, (ed. J. M. Frazier) Marcel Dekker, Inc. New York, pp. 45-83, 1992, and Paine, A.J., Chem.-Biol. Interactions 74:1-31,1990.

Another approach for increasing the survival of cultivated hepatocytes involves co-culturing with feeder cells. Unfortunately, this system encounters

the same difficulties as mentioned before in reference to the Kuri-Harcuch patents.

Thus, while many attempts have been made to produce a long term surviving hepatocyte culture, no one attempt has incorporated such a culture into a test kit which meets all the aforementioned objectives.

SUMMARY OF THE INVENTION

The aforementioned task is met by the present invention which comprises a hepatocyte test kit comprising primary hepatocytes plated in crosslinked collagen coated housings and cultivated in media comprising Chee's Essential Media supplemented with from about 0.2 to about 20 mM glutamine, from about 0.1 to about 10 mM arginine, from about 1.0 to about 100 µM thymidine, from about 1.0 to about 100 µg/ml gentamicin, from about 2 to about 100 mM sodium bicarbonate, from about 1 to about 50 µg/ml insulin, from about 1 to about 50 µg/ml transferrin, and from about 1 to about 50 ng/ml selenious acid. The medium is preferably further supplemented with from about 0.1 to about 10 µM dexamethasone and from about 0.5 to about 2% DMSO at a density from about 25,000 to about 250,000 cells/cm^2. These conditions are referred to herein as CDDP conditions. The test kit may further contain specific assay media and reagents, various toxin standards, quality control information, sample test results and instructions.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 depicts a preferred embodiment of the test kit.

Fig. 2a, b, c, d and e are micrographs illustrating the morphological characteristics of the CDDP-1 hepatocyte.

Fig. 3 depicts cytochrome P450 activity in CDDP-1 cultured hepatocytes at various culture times.

Fig. 4 depicts albumin synthesis results.

Fig. 5 depicts ß-Naphthofavone- and Phenobarbital-mediated inductions of cytochrome P450.

Fig. 6 depicts acetaminophen cytotoxicity results.

Fig. 7 depicts carbon tetrachloride cytotoxicity results.

Fig. 8a, b, c and d depicts cytotoxicity results of numerous chemicals.

Fig. 9 depicts phase II conjugating enzyme activity.

Fig. 10 illustrates a low spontaneous death rate for CDDP-1 cultured hepatocytes.

Fig. 11 depicts the ability to detect slight structural changes by the test kit.

DETAILED DESCRIPTION OF THE INVENTION

The present invention advantageously allows for the prolonged survival of in vitro cultivated hepatocytes and as a result the use of said hepatocytes in a novel kit for testing liver morphology, physiology and pathophysiology. The increased survival rate

allows for morphological and physiological studies alone or in response to drugs, chemical compounds, toxins, etc. Since one of the functions of the liver is the elimination of toxic substances which may take prolonged periods of time, it is very advantageous to have long lived functioning hepatocyte cultures for extended study. The CDDP test kit provides for such cultures.

More specifically, the CDDP test kit may be used to detect compounds which cause or are associated with cytotoxicity, necrosis, cholestasis, carcinogenic potential, unscheduled DNA synthesis, liver growth or regeneration, growth inhibition, hepatocyte differentiation or de-differentiation, hepatic failure, altered hepatic metabolism, viral infectivity, etc.

The CDDP test kit may also be used to study: metabolism of xenobiotics, drugs or endogenous compounds, mechanisms of toxicity, energy metabolism, hepatocyte structure and function, hepatic synthetic functions, hepatocyte differentiation, hepatocyte gene regulation, hepatic enzyme activity, etc.

The possible agents which may be tested with the kit comprise pharmaceutical drugs or drug candidates, new formulations of drugs, biomedical material extracts, industrial or household chemicals, detergents, consumer products and formulations, environmental pollutants, biological fluids, etc.

The test kit contemplated by the present invention comprises primary hepatocytes plated on crosslinked

collagen coated housings and cultivated in media comprising Chee's Essential media (a media produced by GIBCO Laboratories, Life Technologies, Inc., and known as Formula #88-5046) supplemented with from about 0.2 to about 20 mM glutamine, from about 0.1 to about 10 mM arginine, from about 1.0 to about 100 μM thymidine from about 1.0 to about 100 μg/ml gentamicin, and from about 2 to about 100 mM sodium bicarbonate. The medium is preferably further supplemented with from about 1 to about 50 μg/ml insulin, from about 1 to about 50 μg/ml transferrin, and from about 1 to about 50 ng/ml selenious acid. The medium is preferably yet further supplemented with from about 0.1 to about 10 μM dexamethasone and from about 0.5 to about 2% DMSO. Hepatocytes are preferably plated on the housings at a density from about 25,000 to about 250,000 cells/cm^2. Chee's Essential Media supplemented with the aforementioned will hereinafter be referred to as modified CEM. The test kit may further contain specific assay media and reagents, various toxin standards, quality control information, sample test results and instructions.

The primary hepatocytes are prepared and cultured under the following conditions. Hepatocytes are isolated from mature male or female rats using a two-step perfusion method as described by Seglen, P.O., Experimental Cell Research 82:391:398, hereby incorporated by reference. Simply stated, the two-step perfusion method entails removing Ca^{2+} followed by

enzymatic treatment. When treated with collagenase, crude or purified, all the parenchymal tissue is converted to a suspension of cells which is 95% intact.

After isolating the hepatocytes in the aforementioned manner, they are subjected to differential centrifugation o enrich parenchymal hepatocytes. Differential centrifugation purifies the parenchymal cells from non-parenchymal cells.

The substratum is prepared with a crosslinked collagen. Collagens contemplated for use in the present invention include, but are not limited to, the following: rat tail collagen, Vitrogen 100™ (a trademark of Celtrix Pharmaceuticals Inc., Santa Clara, CA), and bovine dermal collagen; Vitrogen, a highly purified bovine collagen preparation, is particularly preferred. The selected collagen may then be poured or coated onto a housing, that may include, for example: flasks, multi-well dishes, petri dishes, etc. Although all generally known laboratory dishes, flasks, and receptables are contemplated, it is preferred that the housings used in the present invention be made from polymethyl pentene or polystyrene, and most preferred that polymethyl pentene housings, such as Permanox™ dishes (available from Nunc, Inc., Naperville, IL), be used.

The culture medium comprises Chee's Essential Media which is supplemented with from about 0.2 to about 20 mM glutamine, from about 0.1 to about 10 mM arginine, from about 1.0 to about 100 μM thymidine,

from about 1.0 to about 100 µg/ml gentamicin, and from about 2 to about 100 mM sodium bicarbonate. It is preferably further supplemented with from about 1 to about 10 µg/ml insulin, from about 1 µg/mg to about 10 µg/ml transferrin, and from about 1 ng/ml to about 50 ng/ml selenious acid. It is preferably yet further supplemented with from about 0.1 to about 10 µM dexamethasone and from about 0.5% to about 2% dimethyl sulfoxide (DMSO).

In a most preferred embodiment, Chee's Essential Media is supplemented with 2 mM glutamine, 1 mM arginine, 41.3 µM thymidine, 26.2 mM sodium bicarbonate, and 50 µg/ml gentamicin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid, 1 uM dexamethasone and 1% DMSO. The particular supplement, and its quantity, is dependent on the assay to be performed; the selection of the supplement is readily ascertainable by one skilled in the art.

Hepatocytes isolated by the aforementioned method are then introduced into modified CEM at a density of from about 100,000 to about 2,000,000 cells/ml. The cell suspension is then pipetted or poured into the aforementioned subtratum-coated housing to achieve a density of from about 25,000 to about 250,000 cells/cm^2. The hepatocytes are then adhered to the housing by incubating them at about 35-40°C for at least 30 minutes, at from about 80 to 100% humidified CO_2:air atmosphere. The media may then be aspirated and replaced with fresh modified CEM culture media to

remove any unattached cells. The media may be replenished at intervals of from about 6 to about 48 hours thereafter. Hepatocytes cultivated under this method will hereinafter be referred to as CDDP hepatocytes.

The test kit is comprised of the CDDP hepatocytes and specific assay media and reagents, various toxin standards, quality control information, sample test results and instructions (see Fig. 1).

Assay media contemplated for use in the present invention include, but are not limited to, the following: Chee's media, William's media, Waymouth's Media, Dulbecco's Modified Eagle Media, RPMI media. A detailed listing of media and their composition can be found in the 1990 Gibco BRL Catalogue & Reference Guide, Life Technologies, Inc., Research Products Division, hereby incorporated by reference.

Reagents contemplated for use with the present invention include chemicals and biological fluids. Biological fluids contemplated include, but are not limited to, serum, plasma, and exudate. The chemical reagents contemplated generally fall into three classes.

The first class of chemical reagents comprises chemicals which are not metabolized but are eventually eliminated from the body in an unaltered form. These chemicals are hereinafter referred to as direct toxins. These may include, for example, drugs such as cadmium

chloride, phalloidin, phosphorous, ethionine, alpha amanitine, etc.

The second class of chemical reagents comprises chemicals which are biotransformed, but whose metabolites have a similar level of toxicity to the parent compound. In this instance, the parent compound is metabolized, but the metabolism is not critical to the toxicity of the compound. For illustrative purposes mention may be made of drugs such as substituted alkyl carbon systems comprising substituted alkanes, haloalkanes, vicinal dihaloalkanes, heteroatom-substituted cyclopropyls; olefinic carbon systems comprising olefins, haloalkenes, dihydrofurans,; aromatic carbon systems comprising; polycyclic aromatic hydrocarbons, halobenzenes, alkenylbenzenes, acetanilides, furans, pyrroles, thiophenes; nitrogen based systems comprising nitrosamines, pyrrolines, aminofluorenes, aromatic amines, azo dyes, hydrazines, nitro compounds; sulfur based systems comprising thiocarbonyls, thiophosphono-compounds; and metal based systems comprising alkyl tins, etc.

The third class of chemical reagents comprises chemicals which are not toxic until they are metabolized, hereinafter referred to as indirect toxins. The present invention is particularly advantageous with this group of chemicals because toxicity can only be determined with fully functioning cells. Illustrative drugs in this class comprise

acetaminophen, cyclophosphamide, aflatoxin B1, alkylnitrosamine, allyl compounds, allyl-isopropyl-acetamide, amino-azo dyes, bromobenzene, carbon tetrachloride and other haloaliphatic compounds, carcinogenic polycyclic hydrocarbons, chloramphenicol, cycasin, ethanol, furosemide, fluroxene, halothane, isoniazid, methoxyflurane ngaione, pyrrolidizine alkaloids, safrole, thioacetamide, urethane, etc.

A more detailed listing of chemicals which fall in the aforementioned three classes may be found in Hepatotoxicity, The Adverse Effects of Drugs and Other Chemicals on the Liver, Zimmerman, Hyman J., M.D., Appleton-Century-Crofts, (1978), incorporated herein by reference. One of the contemplated uses of the test kit of the present invention is for cytotoxicity testing. The general protocol for such testing is described as follows. Specific protocols may vary depending upon the test reagent used.

In general, CDDP hepatocytes are dosed with the selected reagent for a period of time sufficient to provide an effective dose. The effective dose for a particular reagent is a function of sample concentration and dose time, and may be determined by initial range finding studies. Although the effective dose will likely be different for different reagents, the procedures for determining the effective dose of a particular reagent or toxin are well known in the art.

Cellular viability is then tested using one or more of the included assays. These are further discussed below.

As contemplated by the present invention, cytotoxicity testing using the instant test kit will follow generally accepted laboratory procedures including the preparation of separate experimental cultures and controls. Experimental cultures are prepared by aspirating and replacing the culture media in the CDDP housing with fresh modified CEM that has been prewarmed to a temperature of approximately 35°-40°C, and preferably to approximately 37°C. The modified CEM, here, may be prepared without dexamethasone and/or DMSO.

The test reagent is then added to the experimental cultures at a concentration level and for a dosing time sufficient to obtain an effective dose. Preparation of test reagent samples for such purpose is well known in the art. For example, the toxin diazepam may be prepared in an ethanol stock solution at about a 100 mM concentration and added to the culture until final ethanol concentration reaches 1%, e.g. about 1 mM final diazepam dosing concentration. Similarly, cyclophosphamide may be prepared in water at about a 143 mM concentration and then added to the culture to provide a several millimolar concentration; acetaminophen may be added in powder form to modified CEM, incubated until dissolved, to prepare a 20 mM stock solution, and then added to the culture; and

cadmium chloride may be prepared in saline at about a 100 mM concentration, and then further diluted in modified CEM before adding to the culture.

Control cultures may be prepared by aspirating and replacing the culture media with fresh modified CEM, as provided above (for experimental cultures), or with vehicle control. Vehicle controls may contain, in addition to fresh modified CEM, up to 1% ethanol or other alcohol, encapsulated drugs, liposomes, up to 2% DMSO, and up to 10% saline or water.

The cultures are then incubated at approximately 35°-40°C, and preferably at 37°C, in a humidified CO_2:air atmosphere. The cultures are thereafter analyzed with morphological and/or physiological assays to determine toxicity.

Morphological or physiological scoring may be accomplished by a variety of assay including, but not limited to, the following: the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (hereinafter referred to as MTT), for assessing mitochondrial function; the neutral red uptake assay for lysosomal integrity assessment, the lactate dehydrogenase release assay (hereinafter LDH) for plasma membrane integrity assessment, the Alanine Aminotransferase Release assay (hereinafter ALT) for plasma membrane integrity assessment, the glutathione depletion assay, the lipid peroxidation assay, the total protein assay, cytochrome P450 activity assays, albumin assays, bile acid production, secretion or flow

testing, pH testing for assessing changes in energy metabolism, and intracellular calcium levels or distribution.

Two widely used tests for assessing cell death are the MTT and the LDH assays. The MTT viability assay measures reduction of MTT by mitochondrial succinate dehydrogenase in live cells to an insoluble blue formazan salt. The formazan salt is extracted from the cells with isopropanol and the color is quantified by spectrophotometer. Thus, the MTT assay yields relative viability data by giving the percentage of live cells in the test culture versus the percentage of live cells in the control culture.

The following describes the process to be followed when scoring CDDP hepatocytes cultured at a density of 120,000 cells/cm^2 in 60 mm petri dishes.

In the MTT assay, MTT stock solution is prepared by dissolving MTT in phosphate buffered saline (hereinafter PBS) in a ratio of 5 mg:1 ml. The stock solution is further diluted 1:10 in modified CEM culture medium, usually lacking hormones and DMSO, to prepare the working solution.

Where 100% lysis control is desired, the media is first removed from control culture(s); then Triton X100™ (trademark of the Rohm & Haas Co. and generically known as octylphenoxpolyethoxyethanol), preferably a 1% solution, is added to the attached cells and swirled for 10-20 minutes at 50-200 rpm, and preferably for about 15 minutes at approximately 100

rpm. The liquid is then removed and the cells saved for analysis.

In the typical assay, the media is removed from the toxin treated cultures and remaining control cultures and the cells are rinsed with warmed PBS and treated with working solution. It is preferred that the cells remain moist at all times.

The cultures are then incubated at 35°-40°C for 30-60 minutes and preferably at about 37°C for 45 minutes. The MTT solution is then removed without rinsing the cells, because the cells may detach from the dish. Thereafter, isopropanol is added to each culture and swirled under conditions sufficient to solubilize and extract dye from the cells, preferably at about 100 rpm for approximately 10 minutes. Supernatants from each culture are then removed and absorbance at 560 nm, is determined by, for example, a microtiter plate (the reader should be blanked to isopropanol).

The % viable cells may be calculated from the following formula:

$$\% \text{ viable cells} = \frac{\text{ABS 560 sample - blank}}{\text{ABS 560 control - blank}} \times 100$$

A dose response curve may be plotted from the aforementioned formula to obtain an effective concentration at 50% of the dose eliciting the response. The 100% lysis control should show no viable cells.

Alternatively, the MTT assay may be used to quantify the actual number of live cells. To use this methodology, the cells are stained, as described above, followed by the addition of fresh PBS to each culture. The cells are then photographed under a light microscope, without phase contrast. Viable cells are represented by the percentage of the number of stained cells to the total cells in the field.

The LDH assay measures cellular viability as a function of plasma membrane integrity. Lactate dehydrogenase (LDH), a soluble cytosolic enzyme of the glycolytic cycle, is retained in live cells but is released into the surrounding culture media by dead cells through their disrupted membranes. Using LDH reagents, a quantitative spectrophotometric kinetic LDH enzyme assay is performed. The amounts of LDH in cells and the media are measured and compared to give an indication of the proportion of dead cells.

The LDH assay is performed using reconstituted LDH reagent (Sigma Catalog No. 228-10) and Triton X100™. Reconstituted LDH reagent is prepared by adding distilled water to each reagent bottle and warming the bottles to about 30°C. The assay is begun by removing and saving culture media from cells to be tested. Then, a solution of 1% Triton X100™ is added and swirled with attached cells 10-30 minutes at 50 to 200 rpm and preferably for about 10 minutes at about 100 rpm.

A portion of the media or Triton extract is then transferred to a well plate and warmed to about 30°C. Reconstituted LDH assay reagent is added to each well. Using a Molecular Device Place Reader for Dual Kinetics, programmed to the following parameters, read kinetics and subtract any controls from all readings:

Wavelength:	340nm-650nm
Temperature:	30°C
Run Time:	1.5 minutes
Lag Time:	0 seconds
Optical Density:	Limit 0.4
Automix:	once

Cell viability is calculated from the following formula:

% Cell Viability = 100 - % LDH

where,

$$\% \text{ LDH in media} = \frac{Vmax(media)}{Vmax(media)+Vmax(cells)} \times 100$$

and Vmax = maximum velocity of enzyme reaction.

A dose response curve may be plotted for % LDH in media vs. toxin concentration to obtain the effective concentration which elicits 50% of the maximal LDH release.

The invention is further illustrated by reference to the following Examples and Figures.

Example 1

Hepatocyte Isolation and Media and Culture Preparation

Hepatocytes were isolated from the livers of mature male Fischer rat (between 200-250 g, purchased from Charles River Labs, Wilmingham, MA), using the two step collagenase perfusion method as described by Seglen, Exp. Cell Res. 82:391-398 (1973), and as further provided above. Once isolated, the hepatocytes were subjected to differential centrifugation three times for five minutes each at 50 x g.

A substratum was prepared by coating Vitrogen on Permanox plastic petri dishes using morphocarbodi-imide (Aldrich, no. C10, 640-2) as the coupling agent. Vitrogen was diluted to 100 µg/ml in aqueous solution of morpho carbodi-imide (130 µg/ml, final concentration), and the resulting solution was coated on the tissue culture plates at 2 ml/60 mm plate. The plates were thereafter incubated overnight at 37°C. Unbound collagen was thereafter removed by aspiration, and the plates were rinsed once with phosphate buffered saline (PBS) and stored at 4°C until used.

The culture media comprised Chee's Essential Medium (CEM) supplemented with 2 mM glutamine, 1 mM arginine, 41.3 µM thymidine, 26.2 mM sodium bicarbonate, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/mg selenious acid, 50 µg/ml gentamicin, 1 µM dexamethasone and 1% dimethyl sulfoxide (DMSO). This media is hereinafter referred to as CDDP-1 culture media.

Hepatocytes isolated as previously described were therafter diluted into the CDDP-1 culture medium at 6.25×10^5 cells/ml, and 4 mls of cells (2.5×10^6 cells) were seeded onto the aforementioned substratum, the final density being about 120,000 cells/cm^2. The coated dishes were then incubated at 37°C in a 19:1 atmosphere of air:CO_2 and a relative humidity of 95% for 2 hours for cell attachment. Culture media was changed after the attachment period and every 48 hours thereafter using fresh CDDP-1 culture media.

Hepatocytes cultivated under the aforementioned method are hereinafter referred to as CDDP-1 hepatocytes. CDDP-1 hepatocytes were analyzed for morphological and metabolic characteristics, as follows, using light micrographs, scanning electron micrographs and transmission electron micrographs. The results showed that the CDDP-1 hepatocytes have a differentiated morphology that mimics in vivo hepatocytes.

Example 2

Morphologic In Vivo-like Characteristics

Fig. 2a and b are light micrographs illustrating the morphological differences between a prior art primary rat hepatocyte monolayer culture (Fig. 2a) and a CDDP-1 hepatocyte (Fig. 2b). The prior art hepatocyte has a flat and spread de-differentiated morphology, whereas the CDDP-1 hepatocyte has a cuboidal and differentiated morphology which mimics in

vivo hepatocytes. In addition, the CDDP-1 hepatocytes cluster into strands which are reminiscent of the trabecular structures formed by hepatocytes in vivo.

Fig. 2c is a Scanning Electron Micrograph (SEM) illustrating the 3D nature of the cells and the numerous microvilli that stud the surface of the cells.

Fig. 2d is a transmission electron micrograph of a bile canaliculus (BC) formed between adjacent CDDP-1 hepatocytes. Actin filaments visualized by phalloidin rhodamine fluorescence aggregate at the apical (canalicular) surfaces of cell; basolateral surfaces of the cells, identified by fluorescent antibody detection, face the media (not shown). These characteristics show that the novel hepatocytes are polarized in culture and mimic in vivo hepatocytes.

Fig. 2e is a scanning electron micrograph showing fibrin clots synthesized by CDDP-1 hepatocytes. These clots are resistant to 1M urea, a chaotropic agent, suggesting that the CDDP-1 hepatocytes produce a number of clotting factors in vitro. Production and secretion of clotting factors into the plasma is a primary function of hepatocytes in vivo.

Example 3

Metabolic in Vivo-like Characteristics

CDDP-1 hepatocytes were then tested for metabolic characteristics by analysis of cytochrome P450 activity and evaluation of albumin synthesis.

P450 Activity

Fig. 3a depicts cytochrome P450 activity in CDDP-1 cultured hepatocytes at various culture times. The P450 activity was assessed by testing for ethoxyresorufin O-deethylase (EROD) activity, which is a specific function of the P450 1A1/1A2 subfamily of proteins (Fig. 3a). Note the slight induction of the activity after 3 and 6 days.

P450 2B1/2B2 isozyme activity was tested by measuring pentoxyresorufin O-dealkylase (PROD) activity (Fig. 3b). Note only a minimal decline in the activity of this enzyme with culture age.

P450 3A activity was assessed by measuring the hydroxylation of nordiazepam (Fig. 3c). Note the glucocorticoid induction of this enzyme by day 3 and 6.

The activity of P450 2E1 activity was measured as the 6-hydroxylation of chlorzoxazone (Fig. 3d). Note that this activity is maintained for up to 3 days, with maintenance of approximately 60% activity at day 6.

Thus while prior art cultivated hepatocyte systems lose between 50-80% of their P450 activity within the first 24-48 hours of culture, the hepatocytes of the present invention maintained a majority of their P450 activity for at least 6 days in culture. This continued cytochrome activity is of enormous value to the in vitro study of liver physiology in general and more specifically for metabolism and cytotoxicity studies.

Albumin Synthesis

Albumin content was measured in a 24 hour culture medium with an enzyme linked immunosorbent assay (ELISA). The results in Fig. 4 indicate a high level of albumin secretion by these hepatocytes with only a small decrease in albumin synthesis with increased culture age. Albumin production is a liver specific function and is indicative of the differentiated state of these cells in culture.

Example 4

Cell Enzyme Induction and Cytoxicity Testing

ß-Naphthoflavone and Phenobarbital

Using CDDP-1 hepatocytes, cytochrome P450 activity was induced using ß-Naphthoflavone and phenobarbital, respectively. Fig. 5a and b illustrate that like in vivo hepatocytes, the cultured hepatocytes are inducible for cytochrome P450 activity in vivo with these classical inducer chemicals. In contrast, prior art hepatocyte cultures are refractory to induction of P450 enzymes in vitro.

Specifically, Fig. 5a illustrates that the P450 1A1/2 EROD activity is increased 20 times after exposure of the cultures to 25 mM ß-naphthoflavone for a three day period. Similarly, Fig. 5b illustrates that P450 2B1/2 pentoxyresorufin O-dealkylaltion (PROD) activity is induced by a factor of 7 after exposure to 1 mM phenobarbital for 4 days.

The test kit may consequently comprise ß-naphthoflavone and phenobarbital as reagents allowing the user to test for liver cytochrome P450 induction caused by these drugs.

Acetaminophen

Three liver models were compared for their sensitivity to the hepatotoxin acetaminophen (APAP), a drug that requires P450-mediated bioactivation to become a toxic benzoquinone imine metabolite (see Fig. 6). Cells were cultured for 2 days and then treated with APAP for 20 hours and toxicity assessed using the LDH assay. HepG2 cells, a human hepatocellular carcinoma cell line with undetectable levels of constituent P450 activity, are refractory to APAP toxicity. Hepatocytes cultured using conventional techniques (e.g., CDDP-1 conditions without dexamethasone and DMSO) show low levels of P450 activity and a moderate sensitivity to this toxin, whereas CDDP-1 cultures show high levels of P450 activity and are most sensitive to APAP toxicity. The figure illustrates the advantage of using CDDP-1 cells which maintain P450 function for toxicology studies.

Carbon Tetrachloride

The toxicity of CCl_4 is dependent on the maintenance of P450 2E1 activity which activates this chemical to a toxic metabolite. Hepatocytes were treated with 10 mM CCl_4 for 60 minutes and toxicity was

assessed 19 hours later by LDH release (Fig. 7). The control represents spontaneous release of LDH. The results show that CCl_4 is significantly more toxic to CDDP hepatocytes in which P450 activity is maintained than to prior art hepatocytes which did not maintain P450 activity.

Acetaminophen, Chlorpromazine, promazine, diazepam, galactosamine, thioacetamide, $CdCl_2$, naphthalene, ferrous sulfate and amitriptyline

CDDP-1 hepatocytes cultured for 2 days were treated with acetaminophen, chlorpromazine, promazine, diazepam, galactosamine, thioacetamide, $CdCl_2$, naphthalene, ferrous sulfate or amitriptyline, for a 20 hour period. Thereafter the percentage of dead cells was calculated from the release of LDH into the medium or by measuring MTT reduction. Fig. 8a illustrates representative dose-response curves for the LDH assay, as well as the effective concentration for 50% cytotoxicity (EC50) for various chemicals (Fig. 8b). Results of the MTT assay are provided in Fig. 8c (dose response curves) and Fig. 8d (EC50).

This shows that toxicity of a wide range of chemicals with various degrees of toxicity may be measured with the present test kit.

Example 5

Conjugating Enzyme Detoxification Testing

Phase II conjugating enzymes play a major role in many detoxification reactions. It is therefore important to maintain their activity to assess detoxification. Figs. 9a, b, and c illustrate that Phase II enzyme activity also remains intact in the CDDP-1 cultured hepatocytes. The combination of Phase I and Phase II enzyme activity is important to get complete metabolism of xenobiotics and to manifest toxicity of chemicals that require bioactivation.

Specifically, CDDP-1 hepatocytes suffer only a small initial drop in UDP-glucuronyltransferase (Fig. 9a) and glutathione S-transferase activities (Fig. 9b), and recover to near original levels by the third and sixth day of culture.

Fig. 9c illustrates that cellular glutathione levels are close to in vivo levels, 20 nmol/mg protein, after initial seeding and after 3 days in culture. Moreover, glutathione levels remain near 50% in vivo values after 6 days in culture.

Given the aforementioned, the present test kit may advantageously be used to verify whether the liver is capable of metabolizing a particular drug and if so, ascertain the optimal safe level.

Example 6

Low Spontaneous Death Rate and Long-Term Survivability

LDH viability studies were conducted on CDDP-1 hepatocytes. Fig. 10a shows that lactate dehydrogenase (LDH), a cytosolic enzyme released into the medium upon cell death as a function of the disruption of the cellular membrane, is low in the culture medium (light bars) relative to the amounts found inside the CDDP-1 hepatocytes (dark bars). Moreover, the LDH content of the cells remain nearly constant indicating minimal cell loss or death.

The total amount of protein was also analyzed. As shown in Figure 10(b), the total amount of protein associated with each culture of cells remains constant throughout the culture period indicating minimal loss of cells due to cell death. This data demonstrates the advantageous long-term survival of the CDDP-1 hepatocytes, namely at least 10 days.

Example 7

Ability to Detect Slight Differences in Chemical Structures

Since slight changes in chemical structure lead to significant differences in toxicity, it is advantageous for a toxicity test kit to detect slight changes. The results in Fig. 11 demonstrate that the test kit of the present invention does detect small changes. Fig. 11 shows that the novel test kit was able to differentiate between the toxicity of promazine and chlorpromazine,

chemical structures which differ by a single chloride atom on the third ring.

The aforementioned examples show the novel characteristics of the present invention. By way of recapitulation, the present invention comprises a hepatocyte test kit comprising primary hepatocytes cultivated in cross-linked collagen coated housings and cultivated in media comprising Chee's Essential Media supplemented with from about 0.2 to 20 mM glutamine, from about 0.1 to about 10 mM arginine, from about 1.0 to about 100 uM thymidine, from about 1.0 to about 100 µg/ml gentamicin, and from about 2 to about 100 sodium bicarbonate. The media is preferably supplemented with from about 1 to about 10 µg/ml insulin, from about 1 µg/ml to about 10 µg/ml transferrin, and from about 1 ng/ml to about 50 ng/ml selenious acid. It is preferably further supplemented with from about 0.1 to about 10 µM dexamethasone and from about 0.5% to about 2% dimethyl sulfoxide (DMSO).

In a preferred embodiment, Chee's Essential Media is supplemented with 2 mM glutamine, 1 mM arginine, 41.3 µM thymidine, 26.2 mM sodium bicarbonate, 50 µg/ml gentamicin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid, 1 µM dexamethazone and 1% DMSO. The test kit may further contain specific assay media and reagents, various toxin standards, quality control information, sample test results and instructions.

Since certain changes may be made without departing from the scope of the invention as described

herein, it is intended that all matter described in the foregoing specification, including the examples, shall be interpreted as illustrative and not in a limiting sense.

WE CLAIM:

1. An in vitro test kit comprising, primary hepatocytes plated in cross-linked collagen-coated housings and cultivated in media comprising Chee's Essential Media supplemented with about 0.2 to 20 mM glutamine, about 0.1 to 10 mM arginine, about 1.0 to 100 µM thymidine, about 2.0 to 100 mM sodium bicarbonate, and about 1.0 to 100 µg/ml gentamicin.

2. The kit of Claim 1, wherein Chee's Essential Media is further supplemented with about 1.0 to 50 µg/ml insulin, about 1.0 to 50 µg/ml transferrin, and about 1.0 to 50 ng/ml selenious acid.

3. The kit from Claim 2, wherein the Chee's Essential Media is further supplemented with about 0.1 to 10 µM dexamethazone and about 0.5 to 2.0% DMSO.

4. An in vitro test kit comprising, primary hepatocytes plated in crosslinked collagen-coated housings and cultivated in media comprising Chee's Essential Media supplemented with 2 mM glutamine, 1 mM arginine, 41.3 µM thymidine, 26.2 mM sodium bicarbonate, 50 µg/ml gentamicin, 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenious acid.

5. An in vitro test kit comprising, primary hepatocytes plated in crosslinked collagen-coated housings and cultivated in Chee's Essential Media is further supplemented with 2 mM glutamine, 1 mM arginine, 41.3 µM thymidine, 26.2 mM sodium bicarbonate, 50 µg/ml gentamicin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid, 1 µM dexamethazone and 1% DMSO.

6. The kit of Claim 1, wherein the hepatocytes are cultivated at a density from about 25,000 to about 250,000 cells/cm^2.

7. The kit of Claim 1, further comprising reagents.

8. The kit of Claim 7, wherein said reagents comprise nonmetabolizable chemicals, chemicals which are biotransformed, chemicals which are not toxic until metabolized, or biological fluids.

9. The kit of Claim 1, further comprising assays.

10. The kit of Claim 9, wherein said assays comprise the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay, the neutral red uptake assay, the lactatedehydrogenase release assay, the alanine aminotransferase release assay, the glutathione depletion assay, the lipid peroxidation assay, the total protein assay, cytochrome P450 activity assays, albumin assays, and assays for bile acid production, secretion or flow testing, and pH testing.

11. The kit of Claim 1, wherein said housing is selected from the group consisting of a flask, multi-well dish, or a petri dish.

12. The kit of Claim 11, wherein said housing is made from polymethyl penetene or polystyrene.

13. A method for assessing liver morphology, physiology or pathophysiology in vitro, comprising the steps of:

a. exposing primary hepatocytes plated in crosslinked collagen coated housings and cultivated in media comprising Chee's Essential Media supplemented with about 0.2 to 20 mM glutamine, about 0.1 to 10 mM arginine, about 1.0 to 100 µM thymidine, about 2.0 to 100 mM sodium bicarbonate, about 1.0 to 100 µg/ml genamicin, about 1 to 50 µg/ml insulin, about 1 to 50 µg/ml transferrin, about 1 to 50 ng/ml selenious acid, to a reagent; and

b. performing an assay on said exposed hepatocyte cultures to determine the effect of said reagent.

14. The method of Claim 13, wherein the hepatocytes are cultivated at a density from about 25,000 to about 250,000 cells/cm^2.

15. A method for assessing liver morphology, physiology or pathophysiology in vitro, comprising the steps of:

a. exposing primary hepatocytes plated on crosslinked collagen coated housings and cultivated in media comprising Chee's Essential Media supplemented with about 0.2 to 20 mM glutamine, about 0.1 to 10 mM arginine, about 1.0 to 100 uM thymidine, about 1.0 to 100 µg/ml gentamicin, about 2 to 100 mM sodium bicarbonate, about 1 to 50 µg/ml insulin, about 1 to 50 µg/ml transferrin, about 1 to 50 ng/ml selenious acid, about 0.1 to 10 µM dexamethasone and about 0.5 to 2% DMSO, to a reagent; and

b. performing an assay on said exposed hepatocyte cultures to determine the effect of said reagent.

16. A method for assessing liver morphology, physiology or pathophysiology in vitro, comprising the steps of:

a. exposing primary hepatocytes plated in crosslinked collagen-coated housings and cultivated in media comprising Chee's Essential Media supplemented with 2 mM glutamine, 1 mM arginine, 41.3 µM thymidine, 26.2 mM sodium bicarbonate, 50 µg/ml gentamicin, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenious acid, 1 µM dexamethasone and 1% DMSO, to a reagent; and

b. performing an assay on said exposed hepatocyte cultures to determine the effect of said reagent.

17. The method of Claim 13, wherein the assay is selected from the group consisting of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphpenyltetrazolium bromide assay, the neutral red uptake assay, the lactate dehydrogenase release assay, the Alanine Aminotransferase Release assay, the gluathione depletion assay, the lipid peroxidation assay, the total protein assay, cytochrome P450 activity assays, albumin assays, bile acid production, secretion or flow testing, pH testing.

18. The method of Claim 13, wherein the reagent comprises nonmetabolizable chemicals, chemicals which are biotransformed, or chemicals which are not toxic until metabolized; or biological fluids.